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Replacing the Nucleobases in DNA with Designer Molecules

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ABSTRACT
DNA is not only a carrier of genetic information, but it is also a versatile supramolecular scaffold, arranging smaller organic structures into predesigned geometries. Herein are discussed molecular strategies in which the natural DNA bases on the sugar−phosphate backbone are replaced by other molecules. Some of the base replacements under study include fluorophores, ligands for metals, helix stabilizers, and DNA base shape mimics.

A. Introduction: The Properties of Natural DNA

The DNA helix is a molecular assembly that consists of a stacked set of four aromatic heterocycles, held in place by a carbon−oxygen−phosphorus (phosphodiester) repeating scaffold. This scaffold is flexible enough to be remodeled by proteins during transcription of a gene, or by histones in the chromatin assembly, yet it is rigid enough to act as a straight molecule on a length scale of several nanometers, preventing conformation changes that affect biological function until they are induced by proteins. Although we now know of many ways that the natural backbone can be modified, this backbone itself is extraordinarily successful in its array of functions and in its ability to arrange the nucleobases into direct stacked contact.

For most of the functions of DNA, the bases act as the "functional groups" that vary in their information content and their recognition properties, while the backbone remains a constant. Although the natural nucleobases represent a defined set of physicochemical functional properties, chemists are not constrained by natural design. A broader view of possible DNA-like molecules can start with the concept that the natural backbone is one of the best possible scaffolds for arranging organic molecules in a regular, predictable way. Herein are described some strategies for taking advantage of this view in molecular design.

B. What Does the Structural Context Offer?

(i) A Scaffold for Close Molecular Contact. The DNA backbone evolved to hold the flat aromatic DNA bases in a well-organized orientation. In the double-helical structure, the nucleobases are nearly perpendicular to the orientation of the backbone, and they stack on one another much like a roll of coins (Figure 1). In most DNA structures, the helical twist of the polymer requires that the stacked bases are offset somewhat, since each base pair is twisted relative to the next. In essentially all double-stranded nucleic acid structures, the bases are in direct $\pi-\pi$ van der Waals contact throughout the stack, and so the planes of the bases are separated by ca. 3.4 Å, corresponding to the thickness of the π system in an aromatic ring.

In single-stranded DNA, the structure is considerably more flexible, and there is undoubtedly a substantial degree of bond rotation that occurs in the phosphodiester backbone linkages. Nevertheless, there can also be a good deal of ordered structure, caused mostly by the energetically favorable stacking of the bases on one another.[1,2] The cooperativity of even single-stranded helical structure in nucleic acids is no doubt due in large part to the substantial surface area of contact between the column of stacked bases (see Figure 1). It is easy to imagine how movements of one base would affect many of its neighbors. Since many unnatural nucleobases stack more strongly than natural bases,[3] such structure may form in modified DNA-like polymers as well.

(ii) An Easily Synthesizable Repeating Oligomer. From the design and synthesis standpoint, DNA is an attractive system to work with now because decades of previous work by many chemists have made it an easy molecule to assemble.[4] Automated synthesizers can routinely make oligomers in lengths approaching 100 nucleotides, and they can be adapted to incorporate unnatural monomers as well (Figure 2). Moreover, because the synthesis is carried out by an iterative approach, the construction of a chain can easily be carried out in combinatorial fashion, mixing and matching the monomeric components. Finally, modern analytical methods have made characterization of modified oligomeric systems, even when highly charged like DNA, relatively straightforward.

(iii) A Self-Assembling Recognition Motif. Another attractive feature of the DNA backbone is that we can often predict how it will assemble into higher-order structures. We know how to use the Watson−Crick pairing rules to assemble double helices and hairpins, and we can even find new base pairs that can assemble in a selective and stable way. Scientists now know also how to form higher-order helices such as triple, quadruple, and even quintuple helices.[5] By combining helices and junctions, scientists have made impressive self-assembled designed structures with topologies of cubes and other platonic solids.[6] Tertiary structures in nucleic acids are also becoming better understood. DNAs are even capable of being assembled into designed structures that undergo controllable motions, much like molecular machines.[7]

Eric Kool was born in 1960 in Illinois, and received his undergraduate degree from Miami University (Ohio) in 1982. He pursued graduate studies at Columbia University, advised by Ronald Breslow, with whom he studied biomimetic chemistry. He received the Ph.D. in 1988, and continued his training as a postdoc at Caltech with Peter Dervan. In 1990, Kool joined the faculty at the University of Rochester. He was appointed Professor of Chemistry at Stanford University in 1999. His research interests include design of molecular mimics for biological molecules and pathways, and development of new methods for biosensing.

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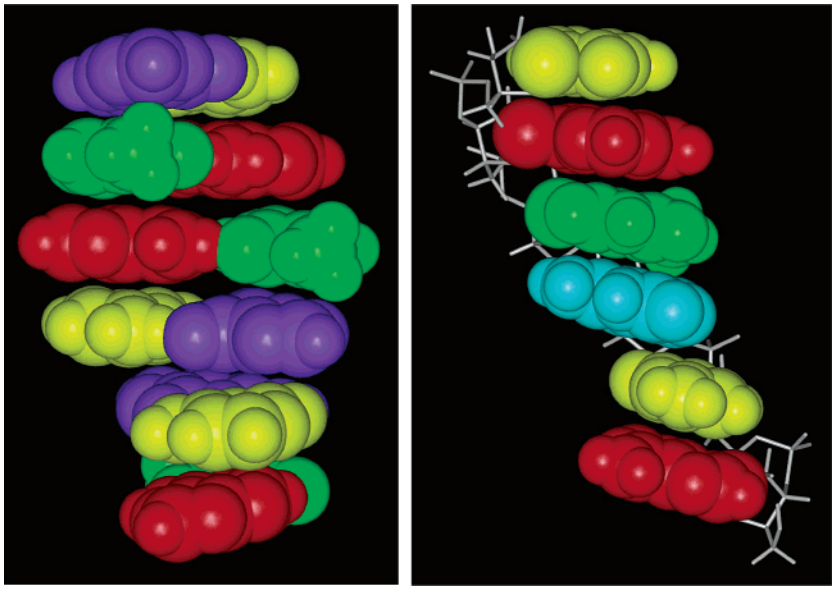

FIGURE 1. Views of how the DNA backbone orients the flat aromatic bases. (a) Space-filling model of the paired bases in double-stranded B-form DNA, with backbone omitted. (b) Model of a single strand of DNA (same conformation) with backbone as stick models and bases as space-filling models. Thanks to Dr. Kevin Guckian (Biogen Inc.) for providing graphics.

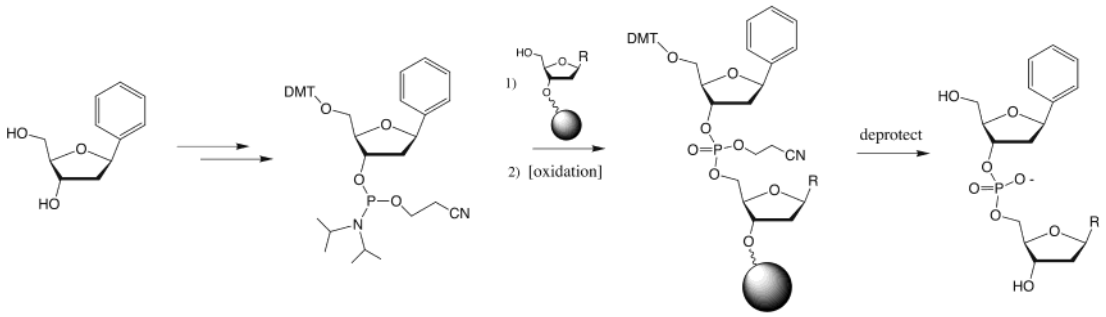

FIGURE 2. Common synthetic strategy used to couple unnatural nucleotides into a designed DNA chain on an automated commercial synthesizer. The first two steps prepare the nucleoside for DNA synthesis; the remaining steps are then carried out on a synthesizer. A benzene base replacement is shown as an example. DMT is dimethoxytrityl.

(iv) A Biopolymer. Of course, the use of DNA as a starting point for molecular design is also attractive because of its manifold biological functions. One can use modified DNAs to probe the mechanisms and structures that govern these functions, such as replication or repair of DNA, or protein−DNA recognition.[8] One can also use modified DNAs as sensors for detecting proteins or nucleic acids. Finally, because there are enzymes whose role is in the replication of natural DNA, we can take advantage of these catalysts to insert nucleosides carrying base replacements into an otherwise natural strand (see below).

C. Why Replace DNA Bases?

Although the four natural bases differ in their structures and functional groups, they are quite limited considering the myriads of organic structures that might be put in their place. DNA bases do not vary greatly in polarity or stacking ability on the wider scale of possible molecules. Neither do they vary much in size or structure, other than offering two types of heterocyclic frameworks. They do not contain strong electrophiles or especially strong nucleophiles, and they generally do not change protonation near neutral pH. They offer (to the first approximation) only four sets of redox potentials. They absorb light only in the ultraviolet range and do so modestly, and they are nearly nonfluorescent. Indeed, one can imagine modifying or expanding many of these properties, and several laboratories are pursuing this now.

D. Classes of Molecular Replacements for DNA Bases

(i) Simple Modifications of Natural Bases. In the past few decades, there have been large numbers of substitutions made on the natural bases. This has been motivated in large part because of the anticancer and antiviral properties of some modified nucleosides. Many simple modifications of DNA bases can impart important biological activity, such as replacing the methyl group of thymine with fluorine, or the oxygen of guanine with sulfur. There is a large body of literature on such nucleoside structural variations; because of space limitations we will not discuss these here and will instead limit the focus to complete replacement of the DNA base with another molecule, which has been a major research strategy in our laboratory.

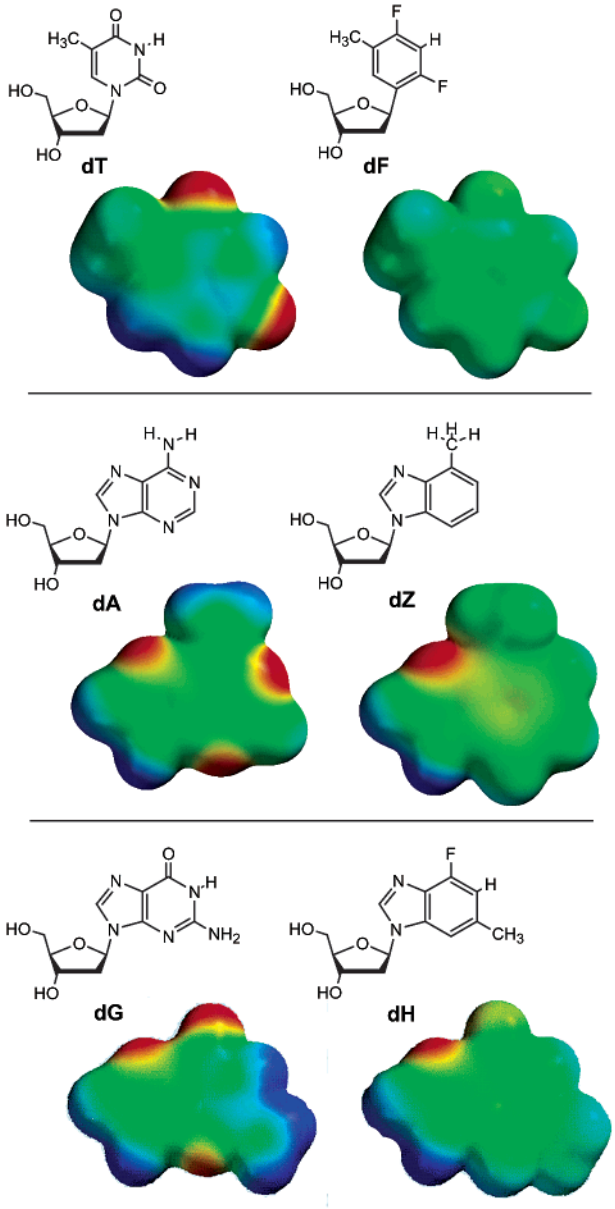

FIGURE 3. Examples of nonpolar isosteres for three natural bases.[8] Complete deoxynucleoside structures are shown; space-filling models have electrostatic potentials mapped on the surface (Spartan software, Wavefunction, Inc.).

Also not included in this Account are natural bases substituted by side chains meant to alter binding properties or to present new functional groups. Again, our current efforts aim instead at making broader, more radical replacements for DNA bases, and so we will not focus on simple substitutions and conjugates here.

(ii) Shape Mimics of Natural Bases. One set of nucleobase replacements that has been particularly useful in our laboratory has been the nonpolar nucleoside isosteres (Figure 3).[9] These structures are the closest possible steric mimics for the natural nucleobases that lack hydrogen-bonding functionality. The design involves replacing oxygen with fluorine and nitrogen with carbon, and keeping aromaticity intact. We have synthesized the four 2′-deoxynucleoside analogues of these compounds[10−12] and have prepared two of the ribonucleoside derivatives as well.[13] Recently, another laboratory also reported ribonucleoside variants.[14]

The difluorotoluene isostere (F) as a nucleoside (dF) makes a nearly perfect mimic of thymidine in the crystalline state and in solution.[15] When substituted in DNAs paired opposite adenine, it adopts a structure identical to that of a T−A base pair.[16] The analogue Z, a benzimidazole isostere of deoxyadenosine, is less perfect in shape but still does a very good job of mimicry as a nucleoside and when substituted in DNAs.[11,17] Structural studies of the G and C analogues, and of the ribonucleoside variants, are underway.

Because of the general lack of polar functional groups, these base replacements are quite nonpolar and hydrophobic. Although fluorine is electronegative, the above analogues containing fluorine do not measurably form hydrogen bonds, even in nonpolar solvents.[18−21]

(iii) Missing DNA Bases. One class of useful nucleoside replacement is the null replacement, where the DNA base is missing. Such compounds (called "abasic") can be used as simple linkers or spacers, or as biological probes of DNA damage and repair. A number of such replacements are now known in the literature.[22,23] We have studied abasic sugars for their pairing properties in DNA opposite other nonnatural nucleosides, as well as for their ability to be replicated by DNA polymerase enzymes. Indeed, we have identified a pairing partner for abasic nucleosides that binds selectively and forms stable base pairs,[24] and that is replicated into DNA efficiently by polymerase enzymes[25] (see below). Coleman has also recently developed a different molecule that can be paired opposite abasic sites.[26]

(iv) Simple Hydrocarbons and Heterocycles. Simple hydrocarbons and heterocycles were among the first replacements for DNA bases (Figure 4). Indeed, the prototypical aromatic hydrocarbon, benzene, was substituted at this position nearly two decades ago.[22] This was designed to act as a nonselective pairing base but was found to be very destabilizing in DNA helices and so was not studied further. This was attributed to poor stacking ability, although we now know that this is incorrect (see below). Our own laboratory made a series of simple aromatic hydrocarbon replacements ranging in size from monocyclic to tetracyclic (benzene, naphthalene, phenanthrene, pyrene).[10,27,28] Simple heterocycles have also been used as replacements for DNA bases, usually with the goal of finding molecules that can pair equally well with all four natural DNA bases. Examples include 3-nitropyrrole,[29] 5-nitroindole,[30] and others.[31]

(v) Fluorescent Species. Fluorescence is an increasingly useful tool for biomolecular analysis and is applied widely in the biosciences. Fluorescence is used in DNA sequencing, in microarrays, in microscopy, and in measurement of binding by polarization anisotropy, among other applications. Fluorescent tags have been most commonly added to otherwise standard DNAs by conjugating common fluorophores (e.g., fluorescein or cyanine) by a linker. Inherently fluorescent DNA bases, such as 2-aminopurine and ethenoadenine, are useful and different because they

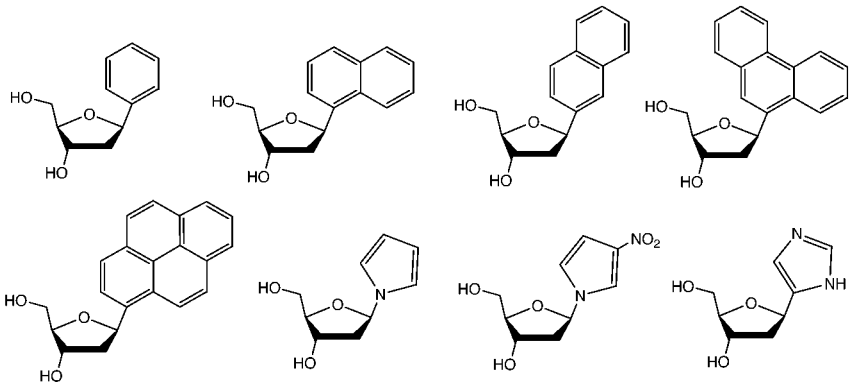

FIGURE 4. Simple hydrocarbon and heterocycle DNA base replacements. Some of these have been used in the study of stacking and as "universal" base analogues.[9,21,26−30]

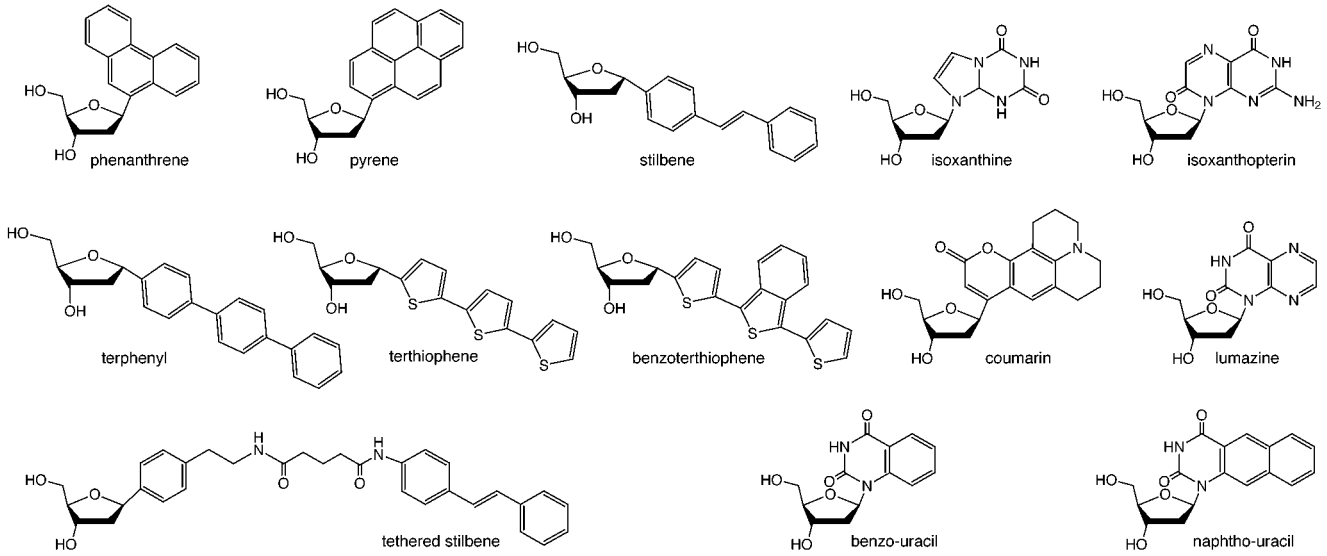

FIGURE 5. Fluorescent DNA base replacements (here termed "fluorosides" in analogy to "nucleosides").[9,25,27,35−40,53] The name of the fluorophore portion is given.

stack with the rest of the DNA rather than hanging by a flexible tether; however, these two are rather weak fluorophores with low absorbances and quantum yields in DNA. Other structural variants of natural nucleosides have also exhibited fluorescence.[32,33]

In a different strategy to offer new luminescence properties, we reported a series of simple fluorescent replacements for DNA bases (Figure 5), based on known hydrocarbon or heterocyclic fluorophores. Examples include stilbene, terphenyl, pyrene, phenanthrene, terthiophene, and benzoterthiophene.[10,28] Some of these have high quantum yields and absorbances, and by making a series of such molecules, we hope to make available a wide range of fluorescence properties, including variable excitation and emission maxima. This has become an active area for investigation recently, and other laboratories have reported base replacement by a coumarin dye,[26] by a tethered stilbene,[54] and by pteridine dyes.[35,36] In addition, further conjugation of natural bases has been carried out, notably with benzo- and naphtho-substituted pyrimidines.[37,38] Coumarin and pyrene nucleotides have proven useful in recent biophysical studies of DNA and enzyme dynamics.[39−41]

One of the most interesting developments in this field has been the study of how multiple dyes interact, using fluorescent DNA base replacements in adjacent positions in a DNA strand. This arrangement of aromatic molecules allows them to interact closely both in the geometric and photophysical sense. Several mechanisms are known for transfer of energy or excitation between one dye and another.[42] For example, when two pyrene nucleosides are aligned in adjacent positions (much like two adjacent DNA bases), they form an efficient excimer complex that emits green light, whereas pyrene alone emits blue.[43] This color change can be used to report on the presence of genetic sequences when two probes bind side-by-side.[44]

More recently, we have described a growing library of nucleosides in which fluorophores replace the DNA base (called "fluorosides", from "fluorophore deoxyribosides").[45] Interactions between these are being studied in a rational way, by multiple substitution in synthetic DNA-like oligomers; for example, we have placed as many as seven pyrene fluorosides in a row into single molecules and have noted increasingly intense excimer emission (Figure 6).[43] Since multiple-dye interactions may be unpredictable, less rational approaches may also be useful.[46] Along this line,

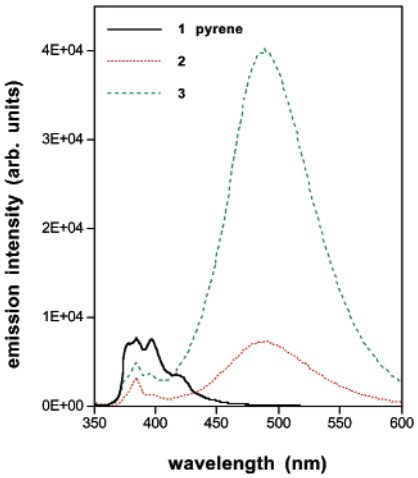

FIGURE 6. Strong excimer fluorescence in pyrene-containing molecules where pyrene replaces the DNA base.[42,43] Numbers (1−3) refer to the numbers of consecutive pyrene nucleosides in the molecule. Note excimer band (490 nm) that arises when two or more pyrenes are adjacent. Excitation is at 341 nm in water.

we have begun making combinatorial libraries of fluoroside monomers.[45] An initial library of monomers was synthesized into an oligomeric string on beads using split-and-pool methods. The resulting beads were imaged under a fluorescence microscope (Figure 9), showing a wide variety of hues and intensities. Analysis of individually interesting polyfluors shows surprising sequence effects on color and intensity. Although this work is in its early stages, it is clear that combining a small set of fluorosides can generate a wide variety of fluorescent species that have properties that single dyes do not.

(vi) Designer DNA Bases and Base Pairs. One of the most active areas of research in DNA base replacements has been in the design of bases that are meant to pair with other non-natural bases. The main goals have been to discover new pairs for selective hybridization and new pairs that might be replicated by polymerase enzymes (Figure 7). One of the earliest approaches was the rearrangement of hydrogen-bonding groups to give altered pairing selectivities, with the isoC-isoG pair studied by Benner[47] as an important example. Work with altered hydrogen-bonding arrangements has been reviewed elsewhere.[48]

In 1995, we described the first base pairs between non-hydrogen-bonding bases, observing selectivity between these hydrophobic molecules.[18] It was notable that these molecules, although they stack strongly,[3,49] are strongly destabilizing to DNA when paired opposite a natural partner, and they were shown to be essentially completely nonselective among these natural partners. This property turned out to be important later when we began to study polymerase enzymes, where different behavior was seen.

In 1997, we reported the first studies with polymerase enzymes, using the nonpolar mimic of thymine, difluorotoluene (F). When F was in a template strand of DNA, we observed that common polymerases such as the Kf enzyme (the Klenow fragment of *E. coli* DNA Pol I) could efficiently insert adenine opposite it.[50] Surprisingly, the

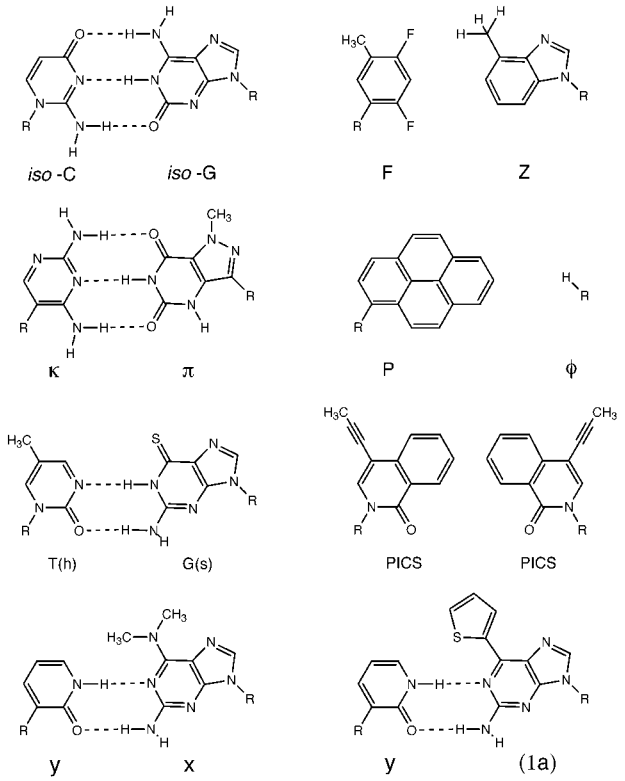

FIGURE 7. Examples of DNA base replacements designed to form stable pairs and/or to be replicated selectively by DNA polymerase enzymes.[17,23,24,49,54,55]

efficiency was not far from that of a natural base pair, which was unexpected because, on its own, F pairs very poorly opposite adenine in the middle of a DNA strand.[16,18] Even more surprising was the high selectivity observed for insertion of adenine opposite F (relative to other nucleotides opposite F), again despite the fact that, on its own, F pairs equally well (which is to say, poorly) with all four bases. This finding provided new insights: first, Watson−Crick hydrogen bonds are not needed to replicate a base pair with high efficiency and selectivity, and second, a polymerase imparts strong steric constraints on a base pair that are more stringent than those imposed by DNA alone.[60]

After that initial finding, we began to explore the generality of the phenomenon; for example, we made the nucleoside triphosphate derivative of F and showed that it was, in fact, selectively inserted opposite an A in the template strand.[51] We synthesized a DNA duplex in which one strand had all thymines replaced with difluorotoluenes.[52] We also showed that an analogue of A (called Z) was also a substrate for polymerases, and that a pair between F and Z was also replicated well.[53] By making an analogue that retains one minor groove hydrogen-bonding group, we pinpointed interactions between polymerases and DNA that are important for extending the DNA through the active site.[54]

More recently, we and others have also studied pairs between molecules that do not resemble natural bases (see Figure 7), based on the idea that hydrogen bonds are not needed to make stable pairs as long as the components stack strongly. The first example of a stabilizing pair

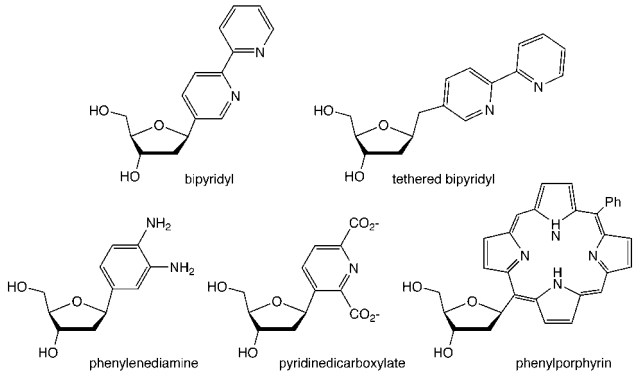

FIGURE 8. Examples of glycosides in which DNA bases are replaced by ligands for metals.[64−67]

lacking hydrogen bonds was the pyrene−abasic pair.[24,25] Recently, the Schultz and Romesberg laboratories have described a number of quite successful examples, including stably hybridizing self-pairs and cross-pairs,[55] and Ishikawa et al. have also contributed new base pairs.[56]

We have proposed that the most important criterion of a polymerase for a successful base pair is that it fit into the tight, rigid active site as would a normal pair.[57−61] Thus, complementarity of size and shape are important, as is stacking ability. Further, if more DNA is to be made, then not only do the bases in a pair need to stack flat opposite one another, but they often need to satisfy minor groove hydrogen-bonding requirements farther down in the enzyme.[54,62] Polymerases place much greater stringency on base pairs than DNA alone does.[61,63,64] To successfully make a complete DNA strand incorporating a new designer base pair, a polymerase must (1) insert the unnatural nucleotide with high efficiency, (2) insert it opposite its intended partner and not others, (3) avoid inserting unintended nucleotides opposite the unnatural base, and (4) efficiently extend more DNA beyond the new base pair.

Despite these impressive enzymatic requirements, the literature now contains a number of successful examples where designer DNA base replacements have been inserted by polymerases successfully into a DNA duplex. The main challenge now is to find base designs (or new enzymes) that can efficiently allow more DNA to be made beyond that point.

(vii) Other Functional Groups: Ligands for Metals. A fascinating new design strategy has been undertaken in a number of laboratories wherein DNA bases are replaced with ligands for metals (Figure 8).[65−69] In some cases, the molecules are designed to be assembled by metals into "base pairs" meant to be isomorphous to a natural pair. In other cases, the designed pairing is meant to be useful as part of a larger non-natural supramolecular architecture. Nucleoside ligands (referred to by Tor as "ligandosides"[67]) in this category include pyridines, bipyridines, anilines, and porphyrins,[69] and no doubt others will be reported soon.

(viii) Increasing Stacking Ability. To better understand the phenomenon called stacking in the context of DNA, we constructed and studied a series of unnatural DNA

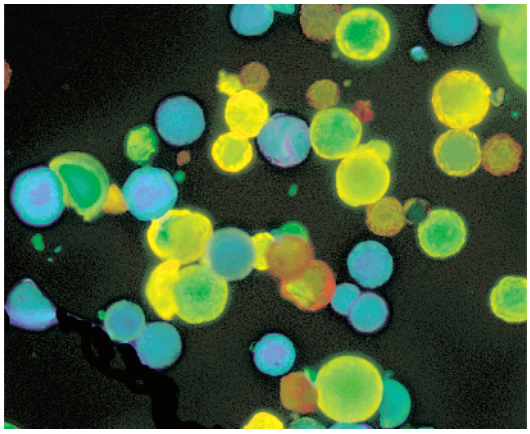

FIGURE 9. Early example of a combinatorial polyfluor library built on a DNA backbone. The variety of fluorescence properties comes from combinations of only four different individual fluorophores.[45]

base replacements and measured their energies of stacking against a DNA helix.[3,49] Several useful conclusions have come from this work. First, the natural DNA bases stack in the order A > G > C,T.[3,49] Second, the DNA bases do not stack very strongly relative to many common organic aromatic molecules. For example, benzene itself stacks better than three of the four DNA bases, and naphthalene better still.[3] Difluorotoluene (F), which has the same size, shape, and polarizability as thymine, stacks much better than thymine, implying that electrostatic attractions are not very important. Overall, size and hydrophobicity play a large part in stabilization of stacking with DNA.[3]

One can use unnatural nucleosides with increased stacking ability to stabilize DNA secondary structure. Placing an unpaired, strongly stacking nucleoside at the end of a helix can stabilize it more than adding a natural full base pair.[3] One can also replace natural nucleosides in looped portions of folded DNAs with unnatural nucleosides that stack more strongly.[70]

Aside from complete replacement of DNA bases, it is worth noting that there are now a number of simple substitutions that one can make onto otherwise natural DNA bases that also stabilize secondary structure, and many of these substitutions increase stacking ability (reviewed in ref 71).

F. Applications: Probing Biomolecular Functions

Some of the most important applications of DNA base replacements have been in basic studies of biological mechanisms. Below are mentioned some of the areas in which base replacements have already been useful. This will be kept brief, since some of this has been reviewed recently.[57,58,61]

(i) Probing Polymerase Mechanisms. Nonpolar nucleoside isosteres have been useful in studying several DNA polymerase enzymes.[58] As mentioned above, we discovered in 1997 that nonpolar base mimics could be replicated by polymerase enzymes, refuting the long-held and widespread belief that Watson−Crick hydrogen bonds

were the main arbiters of base pair synthesis by these enzymes. Since then, it has become accepted that steric and geometric effects in the polymerase active site may be more important.[64,72] However, it still is possible that Watson−Crick bonds do play a significant role,[60] and it remains to be seen (i) how important the relative roles of sterics and hydrogen bonding are, and (ii) how different DNA polymerases vary in this regard. Early studies have been carried out with well-characterized polymerases, but studies are underway now on newer, less well understood enzymes. For example, recently discovered human polymerase iota has the lowest fidelity of any known polymerase, and molecules such as the nonpolar isosteres might help uncover the physical origins of this phenomenon.

Other activities of DNA polymerases are also important to replication and to fidelity, and DNA base replacements are proving their utility here, too. For example, unnatural nucleosides are being used to study the interactions between polymerases and the minor groove hydrogen bond acceptors in DNA.[54] Also under study has been the mechanism of 3′ editing by polymerases, which also adds 1−2 orders of magnitude in the total fidelity of DNA replication.[73]

Finally, other classes of polymerases (aside from DNA polymerases) have also been studied recently. *E. coli* RNA polymerase has been reported in preliminary work[13] to utilize nonpolar replacements of ribonucleotides as substrates. HIV-reverse transcriptase also accepts nonpolar DNA base replacements.[54,74]

(ii) Testing DNA Repair Enzymes. We have undertaken several collaborations to uncover mechanisms by which DNA repair enzymes recognize and distinguish damaged or mismatched DNA from normal DNA.[40,75,76] A general question in this field is how these repair enzymes can find damage without testing every base and base pair in the genome individually. One possibility is that steric and geometric effects play a role, causing a change in the interaction between enzyme and DNA during rapid scanning of the helix. A different hypothesis is that damage generally destabilizes DNA locally, causing repair enzymes to have special affinity there. DNA base replacements such as the nonpolar nucleoside isosteres have been useful in testing such questions. These can retain the natural structure but cause local destabilization, so if the destabilization hypothesis is correct (and early results do seem to support this), then they would be recognized as damage.

G. Future Prospects for DNA Base Replacement

This field remains in its early stages, but the increasing number of creative scientists contributing to it ensure that it will develop rapidly. In the near term, we will see important applications of this approach both to biological studies and to supramolecular chemistry and materials science. In the future, we will see this DNA base replacement approach being applied in even broader ways. When one considers the amazing variations in structure and properties of organic molecules, there is certainly an exciting array of molecular replacements to choose from.

I thank my co-workers for their hard work and enthusiasm; their names appear in the references. I acknowledge support from the NIH (GM52956), the U.S. Army Research Office, and Eli Lilly.

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